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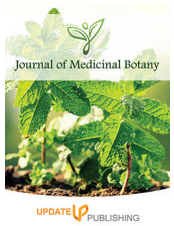

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Antibacterial activities of aqueous and methanol extracts of *Nigella sativa* on some multiple drug resistant diarrheic bacterial agents

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ABSTRACT

Combinations of various antimicrobial agents has been introduced as an extra successful strategy to combat multiple drug resistant infections. This study was undertaken to evaluate the effects of *Nigella sativa* seeds on several multi-drug resistant diarrheic bacterial agents. 30 Stool samples were collected from Lagos State University Teaching Hospital (LASUTH), in Nigeria and standard biochemical tests were performed to confirm the diarrheic isolates. Then, antimicrobial susceptibility testing was done on the organisms, followed by screening the effectiveness of *Nigella sativa* seed extracts on the bacterial agents obtained from the samples. 16 samples tested positive for diarrheic agents *Escherichia coli*, *Escherichia coli* 0157H:7 and *Klebsiella pneumoniae*. The methanolic extracts of *Nigella sativa* showed the highest zone of inhibition of 12mm for *K. pneumoniae* at a concentration of 300mg/dl ,10mm for *E. coli 0157H:7* at a concentration of 200mg/dl, and 10mm for *E. coli* at a concentration of 200mg/dl, respectively. The methanolic extracts showed a much stronger activity than the aqueous extracts of *Nigella sativa* which did not show significant activity towards the diarrheic agents isolated.

KEYWORDS: Antimicrobial agents; *Escherichia coli*; Black seed; Klebsiella pneumoniae; *Nigella sativa*

INTRODUCTION

Increasing rates of antimicrobial resistance have become a worldwide trend predominantly caused by Gram-negative bacteria, especially by the family of the *Enterobacteriaceae*. This family falls within the phylum Proteobacteria, class Gammaproteobacteria and order Enterobacteriales. *Enterobacteriaceae* has become one of the most important causes of nosocomial and community acquired infections. All members of the *Enterobacteriaceae* can cause blood stream and intra-abdominal infections[1]. This may lead to secondary sepsis, a potentially fatal complication mediated by endotoxins. B-Lactams (mainly cephalosporins and carbapenems), fluoroquinolones and aminoglycosides constitute the main therapeutic choices to treat infections caused by these microorganisms.

However, increasing resistance rates to these compounds have been reported in Europe in the last years[2]. During the last two decades, the worrisome trend has been the development of extended-spectrum cephalosporins resistance, e.g. cefotaxime, ceftazidime and ceftriaxone[3]. Such resistance is most often due to the presence of Extended-Spectrum-B lactamases(ESBLs), but may also occur due to plasmid-mediated or chromosomally hyperproduced AmpC B-lactamase[4].Besides resistant to 3rd generation cephalosporin, they are often resistant to different antibiotic families as fluoroquinolones, aminoglycosides and cotrimoxazole[5]. As a result, more patients need antimicrobial treatment using so called 'last resort' agents, as colistin. Carbapenems are considered the best treatment options[6] and the use of carbapenems has led to the rapid selection of carbapenem resistant *Enterobacteriaceae*[7].Antimicrobial treatment options for these multidrug resistant infections are limited. Only a few antimicrobial agents (e.g. colistin, tigecycline, fosfomycin and amikacin) with an uncertain in vivo efficacy and reported toxicity are left to treat these infections.

Enterobacteriaceae are the source of community and hospital acquired infections, they are inhabitants of the intestinal flora and are among the most common human pathogens, causing infections such as cystitis and Pyelonephritis with fever, septicemia, pneumonia, peritonitis, meningitis, and device

associated infection. *Klebsiella pneumoniae* is one the Most common cause of nosocomial infections and it can cause life bacteremia in neonates as well as in adults [8].

Antimicrobial Resistance presents different problems and concerns for humans. The reduction of the efficacy of the treatments is related to the increase of the duration of hospitalization (longer treatments and more invasive), causing higher costs per treatment, and ultimately the complete inefficacity of the drug treatments cause the mortality of patients, particularly for immunocompromised patients, e.g. of cancer therapy or after transplantation [9]. It can be potentially lethal for epidemics (which can be spread globally), and also will be more frequent, and will reach more people, increasing morbidity and mortality and creating a huge impact in the human society. Recent studies that antibiotics affect the normal microbiome, causing different kinds of effects in human health, particularly obesity [10,11]. The usage of antibiotics by children less than two years old has also been linked to an increased risk of early childhood obesity[12]. Another main consequence is that no new effective antibiotics has been developed and introduced in the pharmaceutical market in the last years to cure bacterial infections principally to treat that resistant superbugs[13].

During the recent years, huge number of studies have been carried out, acclaimed medicinal properties emphasized on different pharmacological effects of *N. sativa* seeds such as antioxidant, gastroprotective, anti-anxiety, anti-ulcer, anti-cancer, anti- inflammatory, immunomodulatory and anti-tumor properties, hepatoprotective effect, also gastric ulcer healing, tumor growth suppression, men infertility improvement, cardiovascular disorders, memory improvement, stimulate milk production, protective effects on lipid peroxidation, antibacterial activity, anti- dermatophyte, antiviral activity against cytomegalovirus have been reported for this medicinal plant [14].This present study aims at evaluating the antibiotic resistance patterns among the diarrheic isolates and to determine the effect of *Nigella sativa* on the bacterial agents be it positive or negative.

MATERIAL AND METHODS

Study Centers and Design

A total of 30 patients made up of 20 males and 10 females attending Lagos State University Teaching Hospital (LASUTH), in Nigeria, with diarrhea related illnesses. Faecal samples recruited for this study were collected from male and female subjects of different ages.

Sample Collection and Transportation

30 faecal samples were collected using a sterile universal bottle, the bottles were labelled appropriately. The samples were transported to the laboratory in a cold box and they were immediately refrigerated until analysis were carried out. In the case of *Nigella sativa*, the seeds were obtained from Lagos State University botanical garden and authenticated by the Department of Botany, Lagos state University.

Stool Samples

All collected samples were streaked on basal media and also on differential media like MacConkey, TCBS and XLD. All the plates were incubated at 32^{o}C overnight for further observations.

Isolation and Identification of *Klebsiella* Species and *E. coli* Species

Characterization of the bacteria isolate was based on Standard Microbiological methods.

Stool samples was cultured on Sorbitol MacConkey agar using sterile wire loop, inoculated plates were incubated aerobically at 37^{0}C for 18 to 24 hours.

Bacterial colonies showing typical characteristics of both organisms was subjected to gram's staining and biochemical test.

Identification of the isolates

Identification was done on the basis of morphology, cultural characteristics and biochemical reactions.

Cultural characteristics

After the incubation period, the plates were examined for the size, color edges, side views, odor and surface of the colonies.

Gram Staining

Preparation of smear was carried out on a clean and grease free slide and the smear could air dry and then heat fixed.

The slide was flooded with crystal violet for 1 minute and gently washed off with tap water and flooded with the gram's iodine mordant for 1 minute and gently washed off with tap water.

Furthermore, the slide was decolorized with 95% ethyl alcohol. The reagent was added drop by drop until the alcohol runs almost clear and gently washed off with tap water. Then it was swamped with the counter stain, safranin for 45 seconds and gently washed off with tap water. At the end the slide was blot dried with absorbent paper and examined under oil immersion objective of x100 using a microscope.

Biochemical Test Characterization of the Isolates

Catalase test

The purpose is to detect if the microbes produce catalase, an enzyme capable of destroying the chemical hydrogen peroxide and gaseous oxygen is released. A suspension of 18-24 hours of the test organism was smeared with sterile distilled water on a

clean glass microscope slide. A few drops of hydrogen peroxide (H_2O_2) were added using a dropping pipette. The appearance of gas bubbles caused by the liberation of free oxygen indicated the presence of catalase enzyme (i.e. catalase positive).

Indole production

This test was performed by inoculating peptone water with the test organisms and incubating peptone water overnight. The detection of indole was by the addition of 2ml of kovac's reagent and a cherry-red color at the reagent layer indicated positive indole production. This test is usually done to distinguished *E. coli* from *Klebsiella*.

Triple sugar iron (TSI) test

It is used for the identification of Gram-negative enteric *bacilli* on the basis of lactose and sucrose fermentation and hydrogen sulfides production.

64.52g was suspended into 1000ml of distilled water and heated to boil to dissolve the medium completely. It was sterilized by autoclaving at 121°C for 15mins. 10ml of the medium was distributed into test tubes and allowed to cool in sloped form with a butt of about 1inch long.

Urease test

Urea, a common organic nitrogen source for many microbes, can be hydrolyzed to ammonia and carbon dioxide. The latter produce an alkaline condition in the medium which is indicated by a color change of the pH indicator.

Slants of the Christensen's urea agar medium were inoculated with the isolates and incubated at 35°C for 5-7 days, watching daily for any color changes. The development of color change from yellow to pink showed a positive urease activity.

Citrate utilization

Simmons's citrate medium is a nutrient substrate and an organic synthetic medium that offers ammonium salts as the only source of nitrogen and citrate serving as the only carbon source.

The degradation of citrate leads to alkalization of the medium which is indicated by the pH indicator bromothymol blue changing color from green to deep blue.

Slants of simmon's citrate agar were inoculated with light inoculum of the isolates and incubated at 35°C for 5days. Color change from green to blue indicated a positive result.

Sorbitol test

It is a test used to differentiate *E. coli* 015H7:H7 from other strains of *E. coli*, because other strains are unable to ferment sorbitol. Sorbitol MacConkey agar is used for this test. An inoculum from a pure culture was taken and inoculated on a well-prepared plate of the medium. It was then incubated for 35°c for 24hours, other color change from red to pink indicates the organism is positive

Antimicrobial Sensitivity Testing

Materials: Agar plates with Mueller Hinton medium, Inoculum suspension medium, Normal saline, Antibiotics test discs, Sterile loops and Swabs, Antibiotics disc dispenser/ Sterile forceps.

Procedure

A Standardized Kirby Bauer disk diffusion method utilizing the Mueller Hinton Agar (MHA) (Oxoid, basing stoke, Hampshire, England) plate was performed as per the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Preparation of mueller hinton agar

38g of the powder was suspended in 100ml distilled water and mixed well and swirled properly and mixed thoroughly. It was sterilized by autoclaving at 121°C for 15 minutes and mixed well before pouring into sterile Petri dishes.

Preparation of bacteria suspension

A Large colony (2-3mm diameter) of the bacterium (Pure culture) was inoculated into the 0.85% NaCl solution and making sure that the suspension is homogenous and without clumps of floating bacteria. Mc Far land barium sulfate standard was used to quantitate the suspension and to produce standard inoculum size.

Procedures

For sensitivity antibiotics testing, the procedure of seeding the microbes was followed and the antibiotic multi disc which contained the following antibiotics namely Ceftazidime (30μg), Cefotaxime (30μg), Cefoxitin (50μg), Cefpodoxime (10μg) Ceftriaxone(30μg) and many more were used. The discs were applied either with sterile forceps or antibiotic disc dispenser unto the surface of Mueller Hinton Agar. The plates were incubated at 37°C for 24 hours. After incubation, the susceptibility of the drug was determined by measuring the zone of inhibition of bacterial growth surrounding the antibiotic discs.

The diameter of the zone of inhibition was measured with Vernier Calipers or a thin transparent millimeter scale. An inhibition zone diameter of each antimicrobial test was then measured and interpreted as Resistant (R), Intermediate (I) and Sensitive(S) for each antibiotic.

Preparation of extract of nigella sativa seeds

For extraction process, the *Nigella sativa* seed (locally known as black seed) was crushed in a domestic grinder.

A total of 20g of the black seed powder was weighed and dissolved into 200ml of methanol and distilled water respectively. The water bath was turned on and set to 70^{0}C and the conical flask was put into the water bath and covered. A filter cloth and funnel were used to filter the extract. The filtrates of the extract were dried in the oven at 50^{0}C until the extract gets dried up.

Screening of *Nigella Sativa* Seed Extracts on Bacterial Isolates

Preparation of paper discs

Paper discs of 6mm in diameters was punched from Whatman filter paper. The discs were sterilized in batches of 50 by autoclaving and 10 microliters (10µL) of the prepared concentration (300mg/ml, 200mg/ml, 100mg/ml, 50mg/ml and 25mg/ml) of the methanol and aqueous extracts of *N. sativa* seeds was impregnated to each of the prepared and labelled paper discs to make an extract containing discs of 10mg/discs, 5mg/discs and 1mg/discs.

The extract impregnated discs were placed in an incubator (at 25^{0}c) for 24 hours to dry. After drying, the discs were placed in an appropriate well labelled containers and stored in a refrigerator at 2-8^{0}c.

Preparation of the inoculum

Selected colonies from the identified isolates was picked with sterile wire loop and placed in a test tube containing 5ml of sterile normal saline to make a suspension.

Inoculation of agar plate

The carefully adjusted inoculum suspension could stand for 15 minutes. A sterile cotton swab was dipped into the adjusted suspension, rotated several times and press firmly on the inside wall of the tube above the fluid to remove the excess fluid from the swab. Thereafter, the swab was used to streak over the entire sterile surface of the dried Mueller Hinton agar plate. This process was repeated twice by rotating the plate at approximately 60^0 each time to ensure an even distribution of the inoculum.

Application of discs to inoculated agar plates

The methanolic and aqueous extracts impregnated discs, controls and the standard antibiotics was aseptically placed onto the surface of the inoculated plates. The disc was gently pressed on the agar using sterile forceps to provide uniform contact with the surface. The discs were distributed at least 22mm away from each other and 14mm away from the edge of the plate. The plates were inverted and placed in an incubator set at 35-37^{0}c within 15 minutes after the application of the discs for 18-24 hours (Kirby Bauer 1966). Antibacterial activities were recorded as a clear zone around the discs and were recorded once the zone was greater than 6mm (Clinical and Laboratory Standard Institute, 2019). The zone was measured to the nearest millimeter using ruler held at the back of the inverted petri plate. The petri plates were held a few inches above a black background illuminated with reflected light.

RESULTS

In this study, 30 samples were evaluated, and the isolates was identified by various biochemical tests as shown in Table 1. Of all 30 samples evaluated, 16 stool samples were found to be positive and the bacterial pathogens isolated from the stool samples are; *Escherichia coli* 0157h:7 with the frequency of (8) and *Klebsiella* species (2) and *Escherichia coli* (6) (Table 2). Antibiotic susceptibility test for *Klebsiella pneumoniae*, *E. coli 0157H:7*, and *E. coli* was determined, and Table 3, Table 4 and Table 5 respectively, shows their different resistance and susceptibility pattern against certain antibiotics.

The efficacy of the antibacterial activities at different concentration of the methanolic extract (25mg/ml – 300mg/ml) of *Nigella sativa* on the diarrheic bacterial agents (Figure 1). (*E. coli*, *E. coli 0157H:7*, *Klebsiella pneumoniae*) and the highest amount of concentration (300mg/dl) showed a positive result on *Klebsiella pneumoniae* and at a concentration of 200mg/dl showed a positive result on *E. coli* 0157H:7 and *E. coli* (Table 6).

DISCUSSION

The worrisome trend in the emergence and spread of multidrug resistant diarrheic bacterial agents in both the community and

Table 1: Biochemical Identification for Bacterial Agents Isolated

S/N	GRAM STAINING	TRIPLE SUGAR IRON TEST											
		SLANT	BUTT	H_2S	GAS	LACTOSE	MANNITOL	SORBITOL	UREASE	INDOLE	CATALASE	CITRATE	ORGANISM
1	-	AK	A	+++	+	+	+	+	V	-	+	+	*Citrobacter freundii*
2	-	AK	A	+++	+	+	+	+	V	-	+	+	*Citrobacter koseri*
3	-	A	A	-	+	+	-	-	-	+	+	-	*E. coli*
4	-	A	A	+	+	+	-	+	-	+	+	-	*E. coli 0157:H7*
5	-	A	A	-	+	+	+	+	+	-	+	+	*Klebsiella* spp
6	+	A	A	-	-	+	+	-	+	-	+	+	*Staphylococcus* spp

Legend: TSI = Triple sugar iron, H_2S = Hydrogen sulphide, AK = Alkaline, A = Acid, V = Variable (Either positive or negative), – = (Negative) ,+ = (Positive)

Table 2: Number of bacterial pathogens isolated from stool sample

Type of sample	Nº of samples	Nº of positive samples	Isolates		
Stool	30	16	*E. coli 0157H:7* (8)	*K.pneumoniae* (2)	*E. coli* (6)

Table 3: Antibiotic susceptibility test (AST) for *Klebsiella pneumoniae*

Antibiotics	Ceftazidime (CAZ) 30 μg	Cefuroxime (CRX) 30μg	Gentamicin (GEN) 10 μg	Ceftriaxone (CTR) 30 μg	Erythromycin (ERY)5 μg	Cloxacillin (CXC) 5 μg	Ofloxacin (OFX) 5 μg	Augmentin (AUG) 30 μg	Meropenem (MEM)10 μg
S 12	R	R	R	R	R	R	S	R	S
S 18	R	R	S	S	R	R	R	R	S

Legend: S = *K. pneumoniae* from stool; R = Resistant S = Susceptible

Table 4: Antibiotic susceptibility Test (AST) of *E. coli 0157H:7* isolates

Antibiotics	S2	S5	S6	S11	S12	S13	S17	S18
Ceftazidime (CAZ)30μg	R	R	R	R	R	R	R	R
Cefuroxime (CRX)30μg	R	R	R	R	R	R	R	R
Gentamicin (GEN)10μg	S	S	R	S	R	S	R	R
Ceftriaxone (CTR)30μg	S	S	S	R	S	S	S	R
Erythromycin (ERY)5μg	R	R	R	R	R	R	R	R
Cloxacillin (CXC)5μg	R	R	R	R	R	R	R	R
Ofloxacin (OFX) 5μg	R	R	R	R	R	R	R	R
Augmentin (AUG)30μg	R	R	R	R	R	R	R	R

Legend: S = *K. pneumoniae* from stool; R = Resistant S = Susceptible

Table 5: Antibiotic susceptibility Test (AST) of *Escherichia coli* isolates

Cephalosporin	S1	S3	S8	S9	S10	S14
Ceftazidime (CAZ) 30 μg	S	S	S	S	R	R
Cefotaxime (CRX) 30 μg	S	R	S	R	R	R
Cefoxitin (FOX) 30 μg	S	S	S	S	S	S
Cefpodoxime (CPD) 10 μg	S	S	S	S	S	S
Ceftriaxone (CTR) 30 μg	S	S	S	R	R	R

Legend: S= *E. coli* from stool; R = Resistant, S = Susceptible

Table 6: Table showing the zone of inhibitions of different extracts of *Nigella sativa* on the Bacterial isolates

Nigella sativa concentration	*E. coli*	*E. coli 0157H:7*	*K. pneumoniae*
	M. E A. E	M. E A. E	M. E A. E
25 mg/dL	10 mm N.S	10 mm N.S	7 mm N.S
50 mg/dL	10 mm N.S	10 mm N.S	9 mm N.S
100 mg/dL	10 mm N.S	10 mm N.S	9 mm N.S
200 mg/dL	10 mm N.S	10 mm N.S	10 mm N.S
300 mg/dL	10 mm N.S	10 mm N.S	12 mm N.S

Legend: M. E= Methanolextract, A. E= Aqueous extract, N.S= Not Significant

hospital setting is disturbing, and it is a global treat which is gradually reducing the efficacy of available antibiotics used for the treatment of infectious diseases[15].

However, antibiotic resistance is a real problem, particularly in Nigeria, because of the easy accessibility to cheap

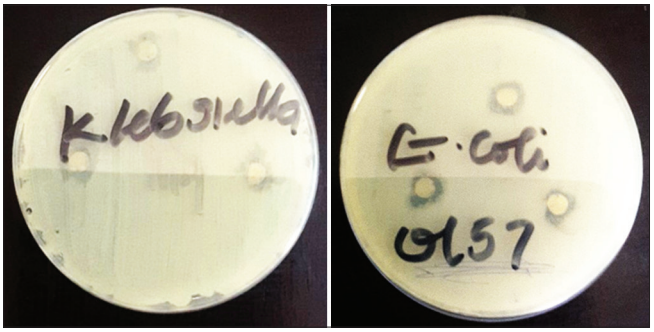

Figure 1: Effect of Nigella sativa on some of the Bacteria Agents isolated

sub-standard drugs in various market corners, which are frequently patronized due to high level of illiteracy and self-medication. Usage of plants as for the treatment of bacterial diarrhea has previously been reported. Masoumeh Navidinia, and Mehdi Goudarzi in 2017[16] studied the anti-bacterial effect of aqueous and ethanolic extracts of *Punica granatum* on *E. coli*, *Shigella sonnei*, *Shigella flexneri*, *Shigella dysenteriae*, *Proteus vulgaris*, *Proteus mirabilis*, and *Citrobacter fraundii*.

In this present study *Nigella sativa* extracts showed great antibacterial properties against diarrheic bacterial agents used. The methanolic extracts of *Nigella sativa* showed strong activity as indicated in Table 6 with a maximum mean value of 12mm for *K. pneumoniae* at a concentration of 300mg/dl, 10mm for *E. coli 0157H:7* with 200mg/dl, and 10mm for *E. coli* at a concentration of 200mg/dl, respectively. In a study conducted in Germany by Seher Nancy Bakal, *et al* in 2017 [17] The antimicrobial properties of Nigella sativa was screened against multidrug-resistant bacteria such as *B. cereus*, *S. aureus Salmonella*, *H. pylori*, *and E. coli* , the results observed in this study collaborated our findings. A similar trend was observed in a study by *Grzegorz Gawron et,al.* in 2019 [18] where they studied the efficacy of Nigella sativa seed extract against methicillin-resistant *Staphylococcus aureus*, and they recorded a very high bactericidal effect against *Staphylococcus aureus* Antifungal activities of Nigella sativa seed extract has also been previously reported, as highlighted in a study by Aftab *et,al.* in 2019[19]. Where methanolic extracts of *Nigella sativa* inhibited the growth of *Fusarium oxysporum* and *Macrophomina phaseolina*

Since the methanolic extracts of *Nigella sativa* seeds in this present study showed greater antibacterial activity, this suggests that methanol is a better solvent for extracting the active ingredients present in the seeds. Traditionally, this plant was prepared by soaking the leaf in alcohol (ogogoro) for days before use to enhance its effectiveness, hence this study justifies the traditional claim of the efficacy of the alcohol extracts of this seed[20].

Previous reports on the phytochemical analysis of this seed showed the presence of biologically active constituents such as thymol, saponins and thymoquinone[21].The presence of these compounds may be responsible for its antibacterial activity, as such further research should be conducted to isolate and characterize the exact components responsible for this feature.

There are many evidences that indicates that the black seed has effective antimicrobial properties against many bacteria, fungi, and viruses and are relatively safe drug with long remarkable history in traditional medicine; it was also more potent that many standard anti-microbial drugs. It is recommended to design and develop novel antimicrobial drugs from *Nigella sativa* seeds. In this line, the mechanism and mode of action of the black seed on the microbial cell (prokaryotic or eukaryotic) and viruses must be well understood, applying new technologies such as nanotechnology may help to reach that goal. The international health authorities must stimulate research and innovation in such promising plant product. Countries and governments are required to provide more financial support and launch research programs for developing new antimicrobial drugs. The major pharmaceutical industries should change its attitude and strategy and invest in natural products of potent antimicrobial effects.

CONCLUSION

Although antibiotics can produce lifesaving benefits, the emergence of multidrug resistance is now a major health worldwide concern. This study revealed the efficacy of methanolic extracts of *Nigella sativa* against some multidrug resistant diarrheic bacterial agents which proved to be quite impressive.

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